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RESISTANCE TO INFECTION WITH *SCHISTOSOMA MANSONI* AFTER IMMUNIZATION WITH WORM EXTRACTS OR LIVE CERCARIAE: ROLE OF CYTOTOXIC ANTIBODY IN MICE AND GUINEA PIGS*

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Abstract. Mice and guinea pigs were immunized with adult and cercarial extracts prepared in different ways. Most animals responded by producing moderate to high levels of cytotoxic antibody; but no correlation with resistance to infection could be detected. Initial experiments with a 3M KCl extract and with cercarial exoantigen produced partial resistance; several attempts to reproduce those results failed. Mice immunized either by live or attenuated cercariae, while developing very low levels of cytotoxic antibody, were highly resistant to challenge infection. These results are discussed in terms of a failure to induce a cooperative immune mechanism required for the action of cytotoxic antibody. It is suggested that homocytotropic antibodies may play a "gatekeeper" role by initiating a reaction that promotes the translocation of serum cytotoxic antibodies and cells into the tissue surrounding the migrating schistosomules.

The development of a laboratory model for the study of acquired immunity to schistosomes has been, and remains, a vital concern for those interested in the control of schistosomiasis. Most laboratory animals, including rodents, rabbits, dogs and monkeys,[1-6] develop significant levels of resistance to challenge after an initial infection. Quite high levels of immunity have been obtained by immunization with gamma or X-ray attenuated cercariae.[7-9] Frustratingly though, extracts of schistosomes do not induce consistent levels of protection. While some workers have reported at least moderate success in immunizing with adult worm extracts and excretory-secretory products,[6,10-13] and with cercarial extracts,[5] an equally convincing series of experiments failed in the attempt to induce resistance to infection.[14-19] Eggs and their extracts have also consistently failed to induce significant resistance.[15,16] This inability to induce reproducible immunity with extracted antigens has limited investigations of the immunology of resistance to schistosome infection. Although many aspects of resistance to schistosome infection are subject to study through the use of models rendered immune by live infection, successful direct immunization with worm antigens would be of particular help in defining the nature of antigens important in resistance, and also a step further along toward a practical vaccine.

In order to develop an immunization procedure that would permit the comparison of different antigen preparations for their efficacy in reducing resistance to infection, we undertook to test many different antigen preparations. This paper is a report of those results and, while we failed initially, at least, to achieve our purpose, we can offer some suggestions that may lead to the solution of this problem.

These experiments also afforded an opportunity to evaluate the role of cytotoxic or lethal antibodies in acquired resistance to *Schistosoma mansoni*. Although these antibodies are quite effective in killing schistosomules and adult worms in

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The animals used in this study were handled in accordance with the provisions of Public Law 89-54 as amended by Public Law 91-579, the "Animal Welfare Act of 1970" and the principles outlined in the "*Guide for the Care and Use of Laboratory Animals*," U. S. Department of Health, Education and Welfare Publication No. (NIH) 73-23.

vitro,[11,20-22] there was sufficient evidence to question their in vivo effectiveness.[11,18] Our results show that resistance to reinfection does not correlate directly with the levels of such antibody activity.

MATERIALS AND METHODS

Infections and worm recoveries

The strain of both *S. mansoni* and its snail host and their laboratory maintenance have been described previously.[23] The exposure of mice to cercariae was accomplished by restraining them in holders and immersing their tails for 1 hour in glass tubes containing an exact number of cercariae. Guinea pigs were first anesthetized with Innovar-Vet (McNeil Laboratories, Inc.), and their abdomens were shaved and fitted with a greased glass chamber. A known number of cercariae were then pipetted into the chamber and allowed to penetrate for 30 minutes.

Autopsies of all hosts were conducted in a similar manner. The animal was injected with a lethal dose of Nembutal (Abbot Laboratories) containing 200 units of sodium heparin. The portal vascular system was then perfused completely with a sodium-citrate solution, and the worms recovered from the perfusate were counted. The livers were also perfused and, in the case of guinea pigs, were examined for worms by squashing the livers between glass plates and examining them with a dissecting microscope. The worm counts were statistically analyzed by the Student's t-test.

Hosts

One strain of mouse was employed: the out bred NIH/Nmri strain maintained at the Naval Medical Research Institute. Female mice 6 weeks of age were routinely used. The Hartley strain of guinea pigs was obtained from the same animal facility.

Antigens

All adult antigens were prepared from adult *S. mansoni* obtained by perfusion from mice 7 weeks after infection. The worms were washed 5 or 6 times with cold 0.85% saline before being frozen and stored at −70° C.

A borate-buffered saline (BBS, pH 7.8) extract of a whole worm homogenate (WH) was prepared by grinding 2 to 4 ml of packed unfrozen worms with a motor driven teflon pestle with the homogenizing tube immersed in an ice bath. The homogenate was then extracted by stirring with a magnetic bar for 18 hours at 4° C. The homogenate was centrifuged at 10,000 × *g* for 1 hour at 4° C, and the supernatant was concentrated under pressure using a PM-10 membrane (Amicon), and stored at −70° C until used.

A freeze-thaw extract of adult worms (FT) was obtained by allowing the previously frozen worms to thaw (4 to 6 ml packed worms in 10 ml BBS) overnight at room temperature. The supernatant was concentrated on a PM-10 membrane, centrifuged at 10,000 × *g* for 1 hour at 4° C and then stored frozen at −70° C.

A procedure designed to extract the membrane antigens of adult worms with hypertonic 3M KCl was adapted from Reisfeld et al.[24] Approximately 5 ml of fresh washed and packed worms were suspended in 100 ml of 3M KCl made up in Hanks balanced salt solution (pH 7.4). The worms were gently agitated for 18 to 24 hours at 4° C, then sedimented by centrifugation (500 × *g* for 10 minutes). The supernatant fluid was exhaustively dialyzed against BBS (pH 7.8) then concentrated to 20 ml by ultra filtration using a PM-10 membrane. The concentrated fluid was then centrifuged at 130,000 × *g* for 1 hour at 4° C and the supernatant was retained.

A cercarial exoantigen (CXO) was prepared by a modification of a procedure described by Fife et al.[25] Cercariae shed into dechlorinated tap water were concentrated (1,000 to 5,000/ml), then held at 4° C for 5 to 7 days. Cercarial suspensions more dilute than this were concentrated before refrigeration. The supernatant was filtered through a 0.45 microfilter, concentrated (5- to 10-fold) on a PM-10 membrane. This preparation was labelled CXO and was used immediately.

Adjuvants

A variety of adjuvants was employed, including Freund's complete (FCA) and incomplete (FICA) adjuvants. In specified experiments, antigen was mixed with 3 mg of alum ($AlNH_4[SO_4]_2$) for intraperitoneal injection. When included, 10^9 *Bordetella pertussis* organisms (Wellcome Lab-

oratories) were injected intraperitoneally. In a single experiment, BCG (Trudeau Institute) was used as an adjuvant. Mice received, at specified intervals, 3×10^6 organisms intradermally, with or without antigen.

Chronic infection

Twenty-five 6-week-old mice were exposed to 50 *S. mansoni* cercariae by the tail immersion procedure. Six months later, 15 infected mice and 10 age- and sex-matched controls were challenged with 50 cercariae. Seven weeks later, all the mice were perfused and their worm burdens were determined. The number of worms recovered from each immunized mouse was corrected by subtracting the mean worm burden of the 10 immunized, unchallenged mice.

Multiple intraperitoneal injections of normal cercariae

Mice were immunized by nine weekly intraperitoneal injections of live cercariae according to the procedure described by Frick et al.[3] Twenty female 6-week-old mice were injected weekly with 10 to 15 *S. mansoni* cercariae. One week after the last injection, 10 of these mice and 10 age- and sex-matched controls were challenged with 50 cercariae by tail exposure. Necropsy was performed on all mice 7 weeks later, and their worm burdens were determined. The worm burdens for each immunized and challenged mouse was corrected as described for the chronic infection experiment.

Ultraviolet irradiation of cercariae

Cercariae were irradiated with ultraviolet light (2,537 Å) for purposes of attenuation by modification of the procedure described by Stafford[26] for *Trichinella spiralis* larvae. Five hundred cercariae in a volume of 2 ml of aged tap water contained in a Syracuse watch glass were placed under a mercury vapor lamp. The lamp emitted 10 μ watts (100 ergs/sec) at 8 cm from the source. The cercariae were irradiated for 3 minutes, then exposed to female 6-week-old mice by the tail immersion procedure. This irradiation dosage had been shown to prevent the maturation of about 95% of penetrating cercariae. Four weeks later immunized mice and appropriate control mice were challenged with 50 cercariae. Necropsy was performed on all mice 7 weeks later, and their worm burdens were determined. As described above, the worm burdens of immunized mice were corrected by subtracting the mean worm count for immunized, unchallenged mice.

Cytotoxic antibody test

Immunized and nonimmunized control animals were bled 3 to 4 days after the last immunization, and challenge exposures were then made 3 to 4 days after bleeding. Mice, after light ether anesthesia, were bled from the retro-orbital plexus using a heparinized capillary pipette (0.3 to 0.5 ml/mouse). Guinea pigs were bled by cardiac puncture (1 ml) without anesthesia. The blood was then spun down at $500 \times g$ for 20 minutes at 4° C; the serum was removed and stored at −20° C until tested.

Cytotoxic antibody levels were measured by a microassay system described previously.[27] Briefly, 50 μl heat-inactivated (56° C, 30 minutes) test serum or plasma, 50 μl guinea pig serum (complement), and 25 μl Earle's lactalbumin hydrolysate medium (GIBCO, Grand Island, New York) containing approximately 50 schistosomules were added in duplicate to flat-bottomed wells of plastic Microtest II plates (Falcon Plastics, Oxnard, California). These plates were then covered and incubated for 20 hours at 37° C in a humidified atmosphere containing 5% CO_2 and 95% air. The proportion of schistosomules which were immotile was then determined by observation with an inverted microscope.

Protein determinations

Protein concentrations were determined by the Lowry method,[28] using bovine serum albumin as the standard.

RESULTS

The results of all immunization experiments with worm extracts are presented in Table 1.

Immunization with whole worm homogenate (WH)

Three groups of female mice 6 weeks of age, along with appropriate controls, were immunized with WH antigens using different adjuvants. As shown in Table 1, immunization failed to confer

TABLE 1

Results of immunization with antigen fractions from Schistosoma mansoni

Immunizing antigen	Animals/ group	Cytotoxic antibody activity ($\bar{x} \pm$ SE) Immunized	Cytotoxic antibody activity ($\bar{x} \pm$ SE) Controls	No. challenge cercariae	No. challenge worms recovered ($\bar{x} \pm$ SE) Immunized	No. challenge worms recovered ($\bar{x} \pm$ SE) Controls
Whole worm homogenate (WH) with:						
FICA*	10 mice	17.6 ± 1.7	1.5 ± 0.3	50	14.4 ± 2.2	10.8 ± 1.8
Alum + *B. pert.*†	10 mice	39.3 ± 1.9	1.1 ± 0.5	50	9.2 ± 1.3	10.1 ± 0.9
BCG‡	10 mice	52.4 ± 5.2	20.9 ± 1.7	50	13.2 ± 1.8	13.3 ± 1.9
Freeze-thaw extract (FT):						
1.0 mg§	6 mice	5.2 ± 1.4	1.0 ± 0.4	50	11.8 ± 2.8	15.6 ± 1.2
2.5 mg§	6 mice	6.1 ± 2.1	1.0 ± 0.4	50	19.3 ± 1.9	15.6 ± 1.2
5.0 mg§	6 mice	46.3 ± 15.1	1.0 ± 0.4	50	17.0 ± 2.9	15.6 ± 1.2
7.5 mg‖	6 g. pigs	45.0 ± 2.4	1.0 ± 0.4	400	65.2 ± 8.1	61.0 ± 11.6
3M KCl extract:						
Exp. #1**	10 mice	16.7 ± 1.4	2.1 ± 0.3	100	22.7 ± 2.4	31.0 ± 2.9 (27%)††
Exp. #2**	10 mice	7.7 ± 1.9	4.7 ± 0.8	50	12.3 ± 2.7	13.8 ± 2.9
Exp. #3‡‡	6 g. pigs	26.1 ± 3.6	2.4 ± 0.3	400	64.5 ± 13.4	61.0 ± 11.6
Cercarial exoantigen (CXO):						
Exp. #1§§	10 mice	15.4 ± 3.5	2.4 ± 0.8	100	30.2 ± 2.9	57.7 ± 3.6 (48%)
Exp. #2§§	10 mice	4.3 ± 0.8	1.6 ± 0.6	50	20.2 ± 1.5	20.9 ± 1.6
Exp. #3§§	10 mice	3.8 ± 0.7	3.7 ± 0.4	50	16.6 ± 2.7	19.7 ± 2.2

* Mice were injected subcutaneously (sq) with 1.0 mg WH in Freund's incomplete adjuvant (FICA) on days 1, 4, and 7. They were injected intraperitoneally (ip) with 1.0 mg WH alone on days 10 and 38; the challenge infection was administered on day 46.

† Mice were injected (ip) with 1.0 mg WH mixed with alum on days 1, 4, and 7. Intraperitoneal injections of 1.0 mg of WH alone were administered on days 10 and 38; the challenge infection was administered on day 46. *Bordetella pertussis* vaccine was given (ip) on day 8.

‡ An intradermal injection of WH (1.0 mg) along with BCG was given on day 1. WH (1.0 mg) was again injected (ip) on days 4, 9, and 18. BCG alone was injected intradermally on day 15. The mice were exposed to challenge cercariae on day 34.

§ Mice were injected (sq) with FT incorporated in Freund's complete adjuvant (FCA) on day 1, and FT alone (ip) on days 14, 18, 21, and 35. The total doses indicated were divided equally between injections. The mice were challenged on day 41.

‖ Each guinea pig was injected (sq) in footpads and nuchal region with 1.5 mg FT in FCA on day 1. Intraperitoneal injections of 2 mg of FT mixed with alum were given on day 13. Injections of 1.0 mg FT (ip) were given on days 16, 18, 21, and 38. The challenge infection was administered on day 44.

** Ten mice were injected (sq) with 500 μg of 3M KCl extract incorporated in FICA on days 1 and 14. 3M KCl extract (500 μg) alone was injected (ip) on days 8, 21, 27, and 31. These mice and controls were challenged on day 36 and perfused 7 weeks later.

†† Figures in parentheses are percent reduction of challenge worms in immunized animals.

‡‡ Six guinea pigs were injected (sq) in the footpads and nuchal region with 1.5 mg of 3M KCl extract in FCA on day 1. Intraperitoneal injections of 1 mg of 3M KCl extract without adjuvant were given on days 16, 18, 21, and 38. The challenge infection was given on day 44, and the guinea pigs were perfused 7 weeks later.

§§ On day 1 500 μg/mouse of CXO in FCA was injected (sq). CXO alone was injected (ip) on day 12 (200 μg), and on days 25 and 33 (100 μg). CXO (200 μg) mixed with alum was injected (ip) on day 12. The mice were challenged on day 46 and perfused 7 weeks later.

any significant protection. Immunization did induce high levels of cytotoxic antibody, however, particularly when incorporated in alum and administered along with an injection of *B. pertussis*.

The third group of mice was immunized with injections of 1 mg WH intradermally (id) simultaneously with an id injection of BCG. After challenge, these mice and their BCG immunized controls were perfused 8 weeks later. Although antigen-injected mice developed a relatively high level of cytotoxic antibody, they exhibited no enhanced resistance to challenge.

Immunization with freeze-thaw antigens (FT)

Groups of mice were immunized with one of three different total amounts of FT extract. The immunized mice and the adjuvant and saline injected controls were perfused 7 weeks after

challenge, and their worm burdens were determined. The results show that FT extract did not induce protection, although the cytotoxic antibody activity in the serum of those mice receiving up to 5.0 mg of FT was quite high.

Similar results were obtained with guinea pigs immunized with FT extract. The immunized guinea pigs and the controls were perfused 8 weeks after challenge. Although a high level of cytotoxic antibody was present at the time of challenge, no resistance to infection was observed.

Immunization with 3M KCl extracts

In an initial experiment, injection of mice with 3M KCl extract induced a significant ($p < 0.05$) degree of resistance to infection. However, when this experiment was repeated (Exp. #2), with the only modification being a challenge of 50 cercariae rather than 100, no acquired resistance was apparent.

Unlike the first experiment, the cytotoxic antibody levels in the mice of the second experiment were not greatly increased. To further analyze the influence of cytotoxic antibody activity on acquired resistance, individual worm burdens and cytotoxic antibody levels from Exp. #1 were compared (Fig. 1). No correlation between this antibody activity and resistance can be demonstrated.

Similar results were obtained with guinea pigs immunized with 3M KCl extract.

Immunization with cercarial exoantigen (CXO)

The first attempt (Exp. #1) to immunize mice with CXO resulted in a significant ($p < 0.01$) resistance to infection. The levels of cytotoxic antibody were relatively low, and did not exhibit any correlation with the percent reduction in worm survival when individual antibody levels were compared with worm counts. Two subsequent attempts (Exp. #2 and #3) to induce a significant level of protection by injections of CXO were unsuccessful, however. The protocols for these experiments varied in that only 50 cercariae were used as a challenge rather than 100.

Immunization with live normal cercariae

High levels of resistance to reinfection were induced by prior infection with normal cercariae (Table 2). The strongest degree of immunity developed in mice chronically infected for 6 months previous to challenge. The levels of cytotoxic antibody at the time of challenge in these mice were very low.

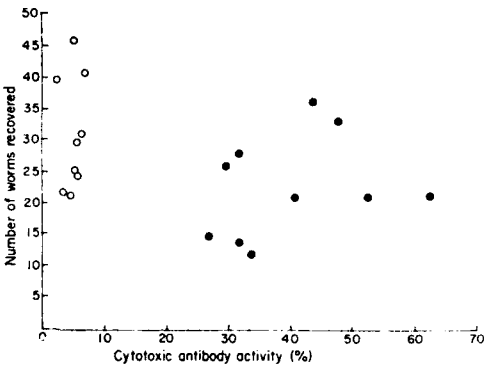

FIGURE 1. Plot of individual mouse pre-challenge cytotoxic antibody levels and post-challenge worm burden. Closed circles indicate mice immunized with 3M KCl extract (Exp. #1); open circles, controls.

Immunization with ultra-violet (UV)-attenuated cercariae

Cercariae attenuated by UV-irradiation induced a high level of resistance to challenge (Table 2). But, as in mice immunized with a live, normal infection, only a slight increase in cytotoxic antibody resulted from this mode of immunization.

DISCUSSION

The immune mechanisms responsible for resistance to schistosome infections are not well understood, although there is substantial evidence indicating that humoral antibodies may be involved in protection against the invasive stage of the schistosome parasite.[6,29-32] Antibodies from infected animals demonstrate cytotoxic and opsonic effects against schistosomules in vitro;[11,20] however, such lethal effects may be artifacts resulting from increased fragility of schistosomules in culture.[18] In the in vivo situation, worms may escape damage by host cytotoxic antibody through adsorption of host antigens,[20,33-36] or by rapid membrane repair.[34]

The lack of correlation between cytotoxic antibody and resistance to infection obtained in this work is in agreement with the results of Sher et al.[18] Our results may reflect failure to induce

TABLE 2

Induction of resistance and cytotoxic antibody in mice with normal and attenuated cercariae of Schistosoma mansoni*

Mode of immunization	Cytotoxic antibody activity ($\bar{x} \pm SE$)		No. challenge worms recovered ($\bar{x} \pm SE$)		% reduction in worm burden	P
	Immunized	Controls	Immunized†	Controls		
Chronic infection with normal cercariae‡	5.0 ± 0.8	2.4 ± 0.6	2.6 ± 1.4	12.0 ± 0.8	78.9	<0.001
Multiple ip injections of normal cercariae§	5.9 ± 1.0	2.6 ± 0.7	3.1 ± 0.9	7.2 ± 1.0	57.3	<0.01
UV-irradiated cercariae‖	4.0 ± 1.2	0.8 ± 0.3	4.0 ± 1.3	11.7 ± 1.4	65.7	<0.01

* Ten animals/group. Each mouse was challenged with 50 cercariae of *S. mansoni*.
† Mean corrected by subtracting mean number of worms from mice immunized but not challenged.
‡ Mice infected 6 months before receiving challenge exposure.
§ Each mouse received nine weekly intraperitoneal injections of 10 to 15 cercariae prior to challenge.
‖ Mice were exposed 4 weeks before challenge to 500 ultra-violet light irradiated cercariae (3 minutes at 100 ergs/sec).

other immunoglobulin classes or subclasses of antibodies required for facilitation or augmentation of cytotoxic immunoglobulin function. This idea has also been proposed by Sher et al.[18]

Homocytotropic and macrophage cytophilic antibodies have been suggested as having potentially important roles in acquired resistance.[29-37] Conceivably, serum cytotoxic antibody may not have sufficient access to the early (vulnerable) stage of the parasite. In that event, translocation of serum antibody to the sites of schistosome migration might be facilitated by a concurrent immediate hypersensitivity response, mediated by one of the classes of homocytotropic or reaginic antibody. If such a response is required for prompt access of other immunoglobulin classes to schistosomules, our failure to induce consistant immunity with worm extracts, even in the presence of high levels of cytotoxic antibody, might be explained by the inability of antigens present to induce sufficient or appropriate homocytotropic antibody. This "gatekeeper" function of homocytotropic or reaginic antibody may be an important component in the overall immune response to tissue invading organisms,[38-42] and the often reported failure to immunize animals with schistosome extracts might be attributed to unsuitable immunization procedures with perhaps inappropriate antigens.

A reagin-mediated increase in serum antibody migration into the tissue sites may result in an enhanced Arthus type reaction. Antibody-antigen complexes are important stimulators of leukocyte infiltration, and IgE-antigen complexes are particularly attractive for eosinophils.[43] The presence of large numbers of leukocytes may be an important element in host resistance; antibody and neutrophilic leukocytes are quite lethal to schistosomula in vitro.[27] Eosinophils have been associated with degenerating schistosomula in the skin of highly resistant rhesus monkeys.[44-46]

Emphasis should also be given to the need to isolate and characterize the many antigens we know to be associated with schistosomes. Our failure to achieve consistent protection with CXO and 3M KCl extracts could simply be due to faulty handling of labile antigens. For example, the release of enzymes during extraction may degrade important antigens. Further, since these crude extracts probably contain considerable amounts of extraneous material,[47-49] not enough of the proper antigen may have been used for immunization. Some antigens present in these complex extracts may also induce counterproductive immune responses, such as blocking factors and enhancing antibodies.[50] Macrophage cytophilic antibody, for example, is easily displaced from macrophages by cytophilic antibody against a second antigen.[51] The injection of a broad spectrum of antigens may have induced adverse antigenic competition.

We hope results reported here will serve to caution others against extrapolating in vitro results to in vivo mechanisms, and to appreciate the complexity of the immune response to schistosome infection.

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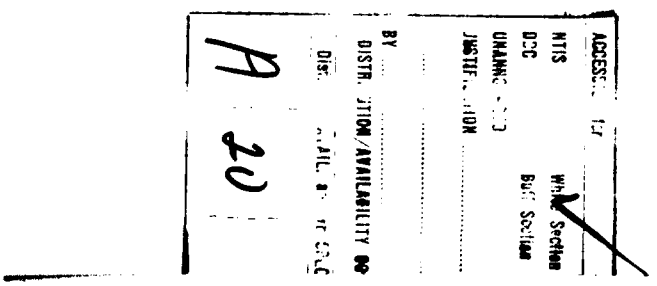

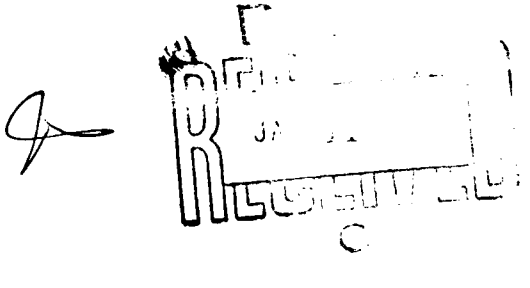

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
Mice and guinea pigs were immunized with adult and cercarial extracts prepared in different ways. Most animals responded by producing moderate to high levels of cytotoxic antibody; but no correlation with resistance to infection could be detected. Initial experiments with a 3M KCl extract and with cercarial exo-antigen produced partial resistance; several attempts to reproduce these results tailed. Mice immunized either by live or attenuated cercariae, while developing very low levels of cytotoxic antibody, were highly resistant to

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challenge infection. These results are discussed in terms of a failure to induce a cooperative immune mechanism required for the action of cytotoxic antibody. It is suggested that homocytotropic antibodies may play a "gatekeeper" role by initiating a reaction that promotes the translocation of serum cytotoxic antibodies and cells into the tissue surrounding the migrating schistosomules.